ELSEVIER

The *Saccharomyces cerevisiae YMR315W* gene encodes an NADP(H)-specific oxidoreductase regulated by the transcription factor Stb5p in response to NADPH limitation☆

Ronald E. Hector[1], Michael J. Bowman[1], Christopher D. Skory[2] and Michael A. Cotta[1]

[1] Fermentation Biotechnology Research Unit, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL 61604, USA
[2] Bioproducts and Biocatalysis Research Unit, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL 61604, USA

Engineered xylose-metabolizing *Saccharomyces cerevisiae* cells grown on xylose show increased expression of *YMR315W* at both the mRNA and protein levels. Additionally, the *YMR315W* promoter contains a putative binding site for the transcription factor Stb5p, which has been shown to regulate genes involved in NADPH production such as *ZWF1*, *GND1* and *GND2*. We hypothesized that Ymr315wp, a conserved protein of unknown function, is an additional source of NADPH in wild-type cells. In this study, we purified histidine-tagged enzyme and determined that Ymr315wp is an NADP(H)-specific oxidoreductase. We also showed that *YMR315W* transcription is regulated by Stb5p in response to diamide induced NADPH depletion. Overexpression of Ymr315wp in BY4727 cells resulted in elevated NADPH levels and increased resistance to diamide. However, the presence of Ymr315wp in cells lacking the oxidative branch of the pentose phosphate pathway resulted in decreased NADPH levels and increased diamide sensitivity. These results suggest that in BY4727 cells Ymr315wp contributes to NADPH production as an alternative source of NADPH.

Introduction

Fermentation technology for the conversion of agricultural biomass into fuel ethanol is hampered primarily by the inability of yeast strains to efficiently utilize pentose sugars. The vast majority of fermentative yeasts are unable to grow on xylose, the most abundant biomass-derived pentose sugar, but some species of *Pichia*, *Candida* and *Pachysolen* have the ability to produce ethanol from xylose. These yeasts rely on the combined activities of xylose reductase (XR) and xylitol dehydrogenase (XDH), which convert xylose to xylitol and subsequently to xylulose, respectively. Most XR enzymes have a preference for NADPH [1], while XDH is typically NAD-linked. This cofactor difference can be problematic as it results in a redox imbalance that can increase the cellular demand for NAD^+ and NADPH [2–5].

The presence of inhibitors formed during biomass pretreatment processes also poses a limitation to efficient fermentation of biomass-derived sugars. Pretreatment is required to enable efficient saccharification to monomer sugars. Most pretreatment processes currently used generate a variety of toxic compounds such as weak acids, furan derivatives (e.g. furfural and 5-hydroxymethylfurfural (HMF)) and phenolic compounds [6]. Many of these compounds severely inhibit fermentation, and it appears that the pentose phosphate pathway (PPP) is required for growth in their presence. The oxidative branch of the PPP functions primarily when cells are grown on glucose, and converts glucose-6-phosphate to 6-phospho-glucono-δ-lactone and then to ribulose-5-phosphate. This route serves to generate biosynthetic pentose sugars and reducing equivalents of NADPH, the latter of which has an important role in the prevention of oxidative stress. Decreased flux through the oxidative branch of the PPP results in

☆ Mention of trade names or commercial products in this article is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Corresponding author: Hector, R.E. (Ronald.Hector@ars.usda.gov)

decreased fermentation of lignocellulosic hydrolysates, presumably due to decreased NADPH availability required to detoxify lignocellulose-derived inhibitors [7]. Furthermore, it was found that several PPP genes are required for resistance to furfural and HMF. Growth in the presence of toxic levels of furfural was improved by the overexpression of glucose-6-phosphate dehydrogenase, encoded by *ZWF1*, which catalyzes the first conversion of the oxidative branch of the PPP [8]. This increased furfural resistance probably occurs because of the excess NADPH made available for pathways associated with stress tolerance.

On the basis of chemostat cultivation studies using glucose/xylose mixtures with cells engineered to express the XR/XDH pathway for xylose fermentation, activity through the oxidative branch of the PPP is significantly increased to supply NADPH for the first step in xylose utilization, xylose reduction to xylitol [9]. It is not entirely clear what pathways are involved in the generation of NADPH when recombinant *Saccharomyces cerevisiae* cells are grown on xylose as the only carbon source. Cells metabolizing xylose could utilize gluconeogenesis to synthesize glucose-6-P for the PPP, and/or rely on alternative sources of NADPH.

Transcript and proteomic studies with recombinant *S. cerevisiae* strains (CEN.PK2 genetic background) expressing *P. stipitis* XR, XDH and elevated endogenous xylulokinase revealed that the open reading frame (ORF) *YMR315W* and resultant protein increased in both aerobic and anaerobic xylose-grown cultures [10,11]. The *YMR315W* ORF encodes a putative 38 kDa protein that is conserved among yeasts. Ymr315wp shows 49%, 41% and 45% similarity to the respective NADP(H)-specific enzymes: glucose–fructose oxidoreductase (GFOR) from *Zymomonas mobilis* [12], xylose dehydrogenase from *Hypocrea jecorina* [13] and 1,5-anhydro-D-fructose reductase from *Sinorhizobium morelense* [14]. The *Z. mobilis* GFOR contains a tightly bound $NADP^+$ and transfers hydrogen from glucose to fructose, producing glucono-δ-lactone and sorbitol. While we did not know if Ymr315wp contained a dissociable $NADP^+$, we hypothesized that as a putative oxidoreductase, Ymr315wp functions to regenerate NADPH. Consistent with this hypothesis, the *YMR315W* promoter region contains a putative Stb5p binding site. Stb5p is a transcription activator that associates with the promoters and regulates the expression of numerous genes required for NADPH production, including many of the PPP genes (e.g. *ZWF1*, *SOL3*, *GND1*, *GND2*, *TKL1*, *TAL1* and *RKI1*) [15]. Additionally, *STB5* deletion strains show increased sensitivity to compounds that increase the cellular demand for NADPH, such as hydrogen peroxide, diamide and furfural [8,15].

The objective of the current investigation was to determine if *YMR315W* was regulated by the transcription factor Stb5p in response to NADPH limitation. We also wanted to determine if deletion or overexpression of Ymr315wp altered NADPH availability. Finally, we purified recombinant His-tagged protein and assayed reductase and dehydrogenase activity to determine cofactor specificity.

Materials and methods

Yeast strains and cultivation conditions

Escherichia coli strains DH10B, TOP10 (Invitrogen, Carlsbad, CA), NEB 5α and NEB 10β (NEB, Beverly, MA) were used for routine maintenance and preparation of plasmids and were grown in LB medium [16]. Yeast strains and plasmids used in this study are listed in Table 1. DNA was transformed into yeast cells using a standard lithium acetate method [17]. Synthetic medium consisted of 6.7 g/l Difco yeast nitrogen base (YNB) (United States Biological, Marblehead, MA), and was supplemented with amino acids [18]. For maintenance of plasmids, media was made without uracil, leucine or histidine as necessary. Synthetic medium was filter sterilized. Sterile glucose was added separately at 20 g/l.

TABLE 1

***Saccharomyces cerevisiae* strains and plasmids used in this study**

Strain	Genotype	Refs
BY4727	*MATα his3Δ200 leu2Δ0 lys2Δ0 met15Δ 0 trp1Δ63 ura3Δ0*	[20]
YRH100	BY4727 *ymr315wΔ::HIS3*	This work
YRH347	BY4727 *zwf1Δ::LEU2*	This work
YRH349	BY4727 *zwf1Δ::LEU2 ymr315wΔ::HIS3*	This work
YRH364	BY4727 [p*YMR315W*]	This work
YRH365	BY4727 [pRS416]	This work
YRH373	BY4727 [pRH280]	This work
YRH405	BY4727 [pRH284]	This work
YRH407	BY4727 [pRH286]	This work
YRH409	BY4727 [pRH280, p*STB5*]	This work
YRH429	BY4727 *stb5Δ::LEU2*	This work
YRH431	BY4727 *stb5Δ::LEU2* [pRH280]	This work
YRH439	BY4727 *stb5Δ::LEU2* [pRH280, p*STB5*]	This work
YRH463	BY4727 [p*STB5*]	This work
YRH485	BY4727 *zwf1Δ::LEU2 ymr315wΔ::HIS3* [p*YMR315W*]	This work

Plasmid		Refs
pCR2.1-TOPO	TA cloning vector	Invitrogen
pET29a(+)	Recombinant His-tagging protein expression vector	Novagen
pRS403	pBluescript II SK+, *HIS3*	[19]
pRS405	pBluescript II SK+, *LEU2*	[19]
pRS406	pBluescript II SK+, *URA3*	[19]
pRS413	pBluescript II SK+, *HIS3*, *CEN6*, *ARSH4*	[19]
pRS423	pBluescript II SK+, *HIS3*, 2μ origin	[19]
pRS416	pBluescript II SK+, *URA3*, *CEN6*, *ARSH4*	[19]
pRH141	pRS413 – P_{PDC1} – MCS – T_{ADH1}	This work
pRH144	pRS416 – P_{PDC1} – MCS – T_{ADH1}	This work
pRH145	pRS423 – P_{PDC1} – MCS – T_{ADH1}	This work
pRH280	YEp353 – $P_{YMR315W}$::*lacZ* [*wild type*]	This work
pRH284	YEp353 – $P_{YMR315W}$::*lacZ* [CaaAGTgATC mutant – m1]	This work
pRH286	YEp353 – $P_{YMR315W}$::*lacZ* [$\Delta C_1 - C_{10}$ mutant – m2]	This work
p*STB5*	pRS413 – P_{PDC1} – *STB5* – T_{ADH1}	This work
p*YMR315W*	pRS416 – P_{PDC1} – *YMR315W* – T_{ADH1}	This work
YEp353	*lacZ* reporter plasmid, *URA3*, 2μ origin	[21]

[ ] indicates plasmids contained in the strain.

Construction of gene deletion strains

Gene deletions were constructed by gene replacement with selectable markers [19]. Each gene deletion was confirmed by PCR

TABLE 2

DNA oligos used in this study

RHFBT#	Sequence
9	5′-ATAGACCCATAATCACTTCTTGGTTCCAAAAAGTAAAGTAATAGCCAAAAAGATTGTACTGAGAGTGCAC-3′
10	5′-AGGGAATCTATCTATTAAGGTTAAAAAAGCGTTACATCGTAAAACGGTAACTGTGCGGTATTTCACACCG-3′
11	5′-TTTCGAACTCGCTCAAACCT-3′
12	5′-CCAAAAGTATACTTATGTGGG-3′
35	5′-CCAACGTGGTCACCTGGCAA-3′
36	5′-GTACCACCGAAGTCGGTGAT-3′
59	5′-GATCGAGTGCTCTATCGCTA-3′
60	5′-CCGTAGTGAGAGTGCGTTCA-3′
139	5′-G*ACTAGTCAT***ATG**TCCCCATTGAACGTCGGT-3′
140	5′-*GGATCC*TCATGGCTGCTCAATTTTAACGT-3′
210	5′-ATCCGAGCGTACAGGGCTAAAAAATTAATACAAAGGTGTAAAAGAAGGACAGATTGTACTGAGAGTGCAC-3′
211	5′-AAGCCGCTGCCGTATAGTATGACGACATGACAAAACTCGGTGAACATATGCTGTGCGGTATTTCACACCG-3′
212	5′-TATAGACAGAAAGAGTAAATCCAATAGAATAGAAAACCACATAAGGCAAGAGATTGTACTGAGAGTGCAC-3′
213	5′-AAAAAAAAATTTCAGTGACTTAGCCGATAAATGAATGTGCTTGCATTTTTCTGTGCGGTATTTCACACCG-3′
216	5′-CGCAGGGAATATAACGACAA-3′
217	5′-CGCTTTAAGAGTTCATGGCA-3′
218	5′-TCGCCAAGGCTATCCCATATA-3′
219	5′-ACATGAGACGACATTCTTTT-3′
237	5′-GC*GGATCC*TAGGTCTGGACACAAAGTATCGTTTC-3′
238	5′-CCA*AAGCTT*TTGCCTTATGTCTG-3′
239	5′-AACAGGGGAT**C**ACT**TT**GTTTATTC-3′
240	5′-GAATAAAC**AA**AGT**G**ATCCCCTGTT-3′
241	5′-CAGGGTTTATTCCCCGGCGGAG-3′
242	5′-CTCCGCCGGGGAATAAACCCTGTTTGAAAATTCAGCAT-3′
249	5′-GG*ACTAGT***ATG**GATGGTCCCAATTTTG-3′
250	5′-G*GTCGAC*TCATACAAGTTTATCAACCCAAGAG-3′

Restriction endonuclease sites are shown italicized and underlined.
Start codons in oligos 139 and 249 are shown in bold.
Nucleotide changes for mutant *YMR315W* promoter m1 (oligos 239 and 240) are shown in bold.

amplification of DNA fragments flanking the disrupted gene. The *STB5* gene was deleted using primers 210 and 211 to amplify the *LEU2* gene and confirmed by PCR amplification from whole cells using primers 216/36 and 35/217 (Table 2). Primers 36 and 35 anneal within the *LEU2* gene, used to replace *STB5*, while primers 216 and 217 anneal to sequences flanking the *STB5* gene. *YMR315W* was deleted using primers 9 and 10 to amplify the *HIS3* gene, and confirmed by PCR amplification from whole cells using primer pairs 11/59 and 60/12. Primers 11 and 12 anneal within the *HIS3* gene, used to replace *YMR315W*. *ZWF1* was deleted using primers 212/213 to amplify the *LEU2* gene and confirmed with primers 232/36 and 233/35. *ZWF1* gene deletion strains were further confirmed by verifying the absence of glucose-6-P (G6P) dehydrogenase activity in clarified cell lysates. G6P dehydrogenase activity was assayed using the enzyme reaction conditions listed below, with 10 mM G6P as the substrate. All DNA oligos (Sigma-Genosys, St. Louis, MO) used in this study are listed in Table 2.

Cloning the STB5 gene, YMR315W gene and YMR315W promoter

DNA fragments for cloning were amplified using *PfuTurbo* Hotstart polymerase (Stratagene, La Jolla, CA). The *STB5* and *YMR315W* genes and the *YMR315W* promoter, were amplified from *S. cerevisiae* genomic DNA (BY4727 [20]) using primer pairs 249/250, 139/140 and 237/238. Adenosine overhangs were added with *Taq* polymerase, and each DNA fragment was cloned into pCR2.1-TOPO (Invitrogen) for DNA sequencing. Error-free DNA fragments were subcloned into yeast expression vectors using restriction endonuclease sites designed into the oligos that were used for amplification.

Construction of wild-type and mutant YMR315W promoter::lacZ gene fusions

The 5′ untranslated region of the *YMR315W* ORF was subcloned, in-frame with the *lacZ* gene, inYEp353 [21] using primer-encoded BamHI and HindIII restriction sites. The *YMR315W* fragment contained the promoter, plus 130 bp of the open reading frame. To generate mutant promoters, each mutant promoter was PCR-amplified to generate two separate overlapping fragments. Mutant m1 fragments were amplified using primers 237/239 and 240/238. Mutant m2 fragments were amplified using primers 237/241 and 242/238. These amplified fragments contained the primer-encoded nucleotide changes, as well as significant homology to

the other fragment to allow 'fusing' of the two fragments together in a second PCR step. To generate the full-length mutant promoter, the second amplification was performed using a 50:50 mixture of the first two PCR products as template DNA, and primers 237/238. Each full-length mutant promoter fragment was cloned into pCR2.1-TOPO for DNA sequencing. Error-free DNA fragments were subcloned into YEp353 as before.

Transcriptional activity assays

The Beta-Glo Assay system (Promega, Madison, WI) was used to determine the level of transcriptional activity from promoter::*lacZ* fusions. Log phase (0.2–0.8 OD_{660}) cultures were diluted in fresh medium to a final $OD_{660} = 0.004$. Assays were started by adding 50 μl of diluted cells to 50 μl of Beta-Glo reagent, mixed thoroughly, and incubated at room temperature. The Beta-Glo reagent contains a detergent that lyses cells to release the β-galactosidase present. Using these conditions, activity measurements were stable from 60 to 120 min. All assays were performed in 96-well, opaque (white), flat-bottomed microtiter plates. At 90 min the samples were read using the luminescence mode of a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). β-Galactosidase activities reported in the figures are based on the Relative Light Units (RLU) measured. Each assay was initiated using the same amount of cell mass to minimize variation due to differing cell concentrations. Assays were repeated at least in triplicate.

Transcriptional activity in diamide-stressed cells was performed as above, with the following changes. Cultures were grown to an $OD_{660} = 0.4$ and an equal amount of cell culture was mixed with 3.6 mM diamide (MP Biomedicals, Solon, OH) (diluted in medium), resulting in a final diamide concentration of 1.8 mM. Cells were incubated for 30 min at 30°C, shaking at 150 rpm, and processed as above.

Diamide sensitivity assays

Diamide sensitivity assays were performed essentially as in [22]. Log phase cultures were diluted to a final $OD_{660} = 0.001$ into 2× concentrated medium. To initiate diamide stress, 100 μl of diluted cells was added to 100 μl of sterile water containing varying amounts of diamide. The microtiter plates were sealed with a Corning sterile breathable membrane (Corning, NY) and incubated at 30°C for 44 h, shaking at 150 rpm. Before reading the OD_{660} in a Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA), the microtiter plates were mixed to thoroughly resuspend any cells that might have settled. All strains and each condition tested were performed in at least triplicate and mean values are reported.

NADP(H) measurement

Enzymatic cycling assays to determine $NADP^+$ and NADPH amounts were performed according to the $NADP^+$/NADPH Quantification Kit (BioVision, Mountain View, CA), with the following modifications. Cells were grown to $OD_{660} = 0.8$ and 3 ml of cells were removed and added to 11 ml of cold methanol (maintained in a dry-ice ethanol bath) to quickly quench cellular metabolism. The cells were centrifuged for 1 min at −9°C (3500 × *g*). The supernatant was removed and the cells were resuspended in 500 μl of cold methanol and transferred to a microfuge tube. The cells were centrifuged again (16,000 × *g*) for 30 s at 0°C and the supernatant removed. The cell pellet was freeze-dried for 30 min and stored at −80°C overnight. The dried cell pellet was resuspended in 300 μl of Extraction Buffer (BioVision) and added to 200 μl of prechilled glass beads (Sigma, St. Louis, MO). Cells were lysed by three rounds of vortexing for 10 s followed by incubation on ice for 30 s. The lysed cells were centrifuged (16,000 × *g*) for 1 min at 0°C and the clarified cell lysate was transferred to a new prechilled microfuge tube. Using this lysate, NADP(H) amounts were measured according to the manufacturer's protocol (BioVision). Protein amounts for each lysate were determined using Quick Start Protein Bradford reagent (BioRad) and bovine serum albumin. Cofactor values were normalized to the amount of protein in the cell lysate and are reported as nanomoles of cofactor per milligram of protein. All cofactor values were determined in triplicate.

Recombinant His-tagged Ymr315wp purification

To generate His-tagged recombinant Ymr315wp, the *YMR315W* open reading frame was cloned into the expression vector pET29a(+) (Novagen, Madison, WI) and transformed into *E. coli* BL21 cells for expression. Recombinant protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and protein was isolated and purified under native conditions using the Ni-NTA Fast Start kit (Qiagen, Valencia, CA), according to Manufacturer's instructions. Purified protein was concentrated and diluted in buffer containing 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, 0.5 mM DTT and protease inhibitors. Protease inhibitors were diluted according to the Manufacturer's instructions (Complete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN).

Enzyme assays

Reductase activity was assayed in buffer containing 33 mM sodium phosphate, pH 7.0, 10 mM $MgCl_2$ and 0.15 mM NADPH or NADH. Dehydrogenase activity was assayed in buffer containing 33 mM sodium phosphate, pH 8.0, 10 mM $MgCl_2$ and 1 mM $NADP^+$ or NAD^+. Reactions were started by the addition of substrate to a final concentration of 10 mM. Reactions were monitored by following NAD(P)H absorbance at 340 nm using a Benchmark Plus microplate spectrophotometer (BioRad) and activity was determined in nmol/min/mg of protein. The molar absorption coefficient, ε_{340}, was 6.22 mM^{-1} cm^{-1} for NAD(P)H. Values are reported as relative activity compared to DL-glyceraldehyde reduction (38 nmol/min/mg) and L-arabinose oxidation (28 nmol/min/mg).

Analytical methods

Extracellular metabolites were analyzed using high-performance liquid chromatography (HPLC). Culture samples were clarified by centrifugation (16,000 × *g* for 10 min) to remove cells. Supernatant solutions were maintained at −80°C before analysis. The separation system consisted of a solvent delivery system (P2000 pump, Thermo Scientific, Waltham, MA) equipped with an autosampler (717 plus, Waters Chromatography Division, Millipore Corp., Milford, MA), a refractive index detector (410 differential refractometer, Waters) and a computer software based integration system (Chromquest 4.0, Thermo Scientific). The ion-moderated partition chromatography column (Aminex HPX-87H with

Cation H micro-guard cartridge from Bio-Rad Laboratories) was used. The Aminex HPX-87H column was maintained at 65°C, and the products were eluted with 10 mM HNO_3 prepared in Milli-Q (Millipore) filtered water at a flow rate of 0.6 ml/min. Peaks were detected by refractive index and were identified and quantified by comparison to retention times of authentic standards (glucose, glycerol, ethanol, acetate and erythritol). Product concentrations are reported as (g/l).

For gas chromatography–mass spectrometry (GC–MS) analysis, 20 μl of culture supernatant was dried *in vacuo*, followed by addition of 200 μl SylonBFT (BSTFA:TCMS 99:1, Supelco):Pyridine (1:1) and heating at 65°C for 30 min. After cooling, 1 μl was injected on a DB5XB column (30 m × 0.25 μm, Shimadzu) with a 1:10 split ratio. After an initial 3 min of isothermal elution at 100°C, elution was effected by use of a thermal gradient from 100 to 300°C at a rate of 10°C/min. A 9-min cut-off was used to avoid MS fouling by solvent peaks. The inlet temperature was set to 250°C. Erythritol was identified by comparison to an authentic standard.

Statistical analyses

Probability analyses were performed using the Student's *t*-test with a two-tailed distribution, compared to the appropriate control strain. Values with $P < 0.05$ were considered significant for this study.

Results and discussion

YMR315W transcription requires Stb5p

The promoter region for the *YMR315W* gene contains a putative binding site (CGGAGTTATC) for Stb5p at −180 nucleotides from the ATG codon (Fig. 1A) [23]. Stb5p, a transcription factor that regulates several PPP and other NADPH producing genes, has been shown to have both transcriptional activation and repression activities, and has also been shown to interact with the consensus sequence CGGnstTAta [15,23]. To determine if transcription of *YMR315W* was regulated by Stb5p, we assayed transcriptional activity from the *YMR315W* promoter. The *YMR315W* promoter was fused to the *lacZ* gene to generate the reporter plasmid pRH280 (Fig. 1A). Transcriptional activity, measured as β-galactosidase activity, was determined using the parent strain, BY4727, and YRH431 cells with the *STB5* gene deleted. We also generated two promoters with mutations to nucleotides shown to be important for Stb5p binding [15]. Mutant promoter m1 changed the consensus nucleotides to CaaAGTgATC, where altered nucleotides are shown in lower case. Mutant promoter m2 deleted the entire putative Stb5p binding site. Deletion of the *STB5* gene resulted in a 76% ($P = 0.0002$) decrease in transcriptional activity (Fig. 1B, bar 1 versus 3). Mutation or removal of the putative Stbp5 binding site also significantly reduced transcription (bars 5 and 6 versus 1). Replacement of Stb5p by expression from a low-copy vector (p*STB5*) in *stb5*Δ cells restored transcriptional activity to wild-type levels (bar 3 versus 4), while increasing Stb5p expression in the parent strain resulted in a 38% ($P = 0.002$) increase in activity over normal levels (bar 1 versus 2). These results showed that Stb5p is a transcriptional activator of the *YMR315W* gene and further suggested that transcription is regulated in a dose-dependent manner by Stb5p, through its interaction with the consensus sequence at −180.

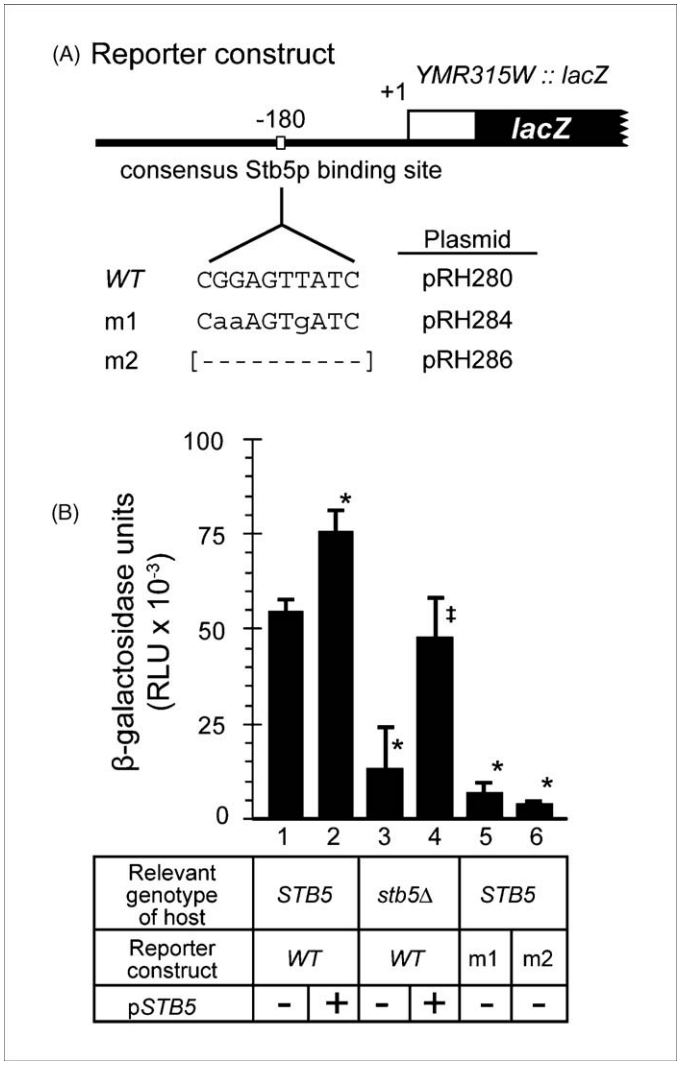

FIGURE 1

Stb5p mediated transcriptional activation of the *YMR315W* promoter. **(A)** Representation of the *YMR315W* promoter fused to the *lacZ* gene. A putative binding site for Stb5p is found at −180 nt. Changes to the putative binding site to create mutant promoters (m1 and m2) are shown below the consensus sequence. **(B)** β-Galactosidase activities were determined for *STB5* and *stb5Δ* cells with and without Stb5p expression from the low-copy vector p*STB5* (bars 1–4). Transcription was also assayed from the mutant promoters in *STB5* cells (bars 5 and 6). β-Galactosidase activities are reported as relative light units (RLU × 10^{-3}) and are the average values for ≥3 independent cultures. Error bars represent the standard deviation. *Value is significantly different from the parent strain, Bar-1 ($P < 0.05$). ‡Value is significantly different from the *stb5Δ* strain ($P = 0.0005$), bar 3 versus 4.

YMR315W transcriptional activation by diamide is mediated through Stb5p

Diamide, a thiol-oxidizing agent, rapidly oxidizes glutathione (GSH) to glutathione disulfide (GSSG) [24]. GSH has various cellular activities, including acting as a radical scavenger protecting the cell from oxidative damage. Consequently, the ratio of GSH/GSSG is indicative of the level of oxidative stress within a cell and cells typically maintain a high GSH/GSSG ratio [25]. During exposure to diamide, to maintain this high GSH/GSSG ratio, glutathione reductase (*GLR1* in *S. cerevisiae*) uses NADPH to reduce GSSG to GSH, thereby depleting NADPH levels in diamide-treated cells. The ability of yeast cells to survive diamide exposure depends

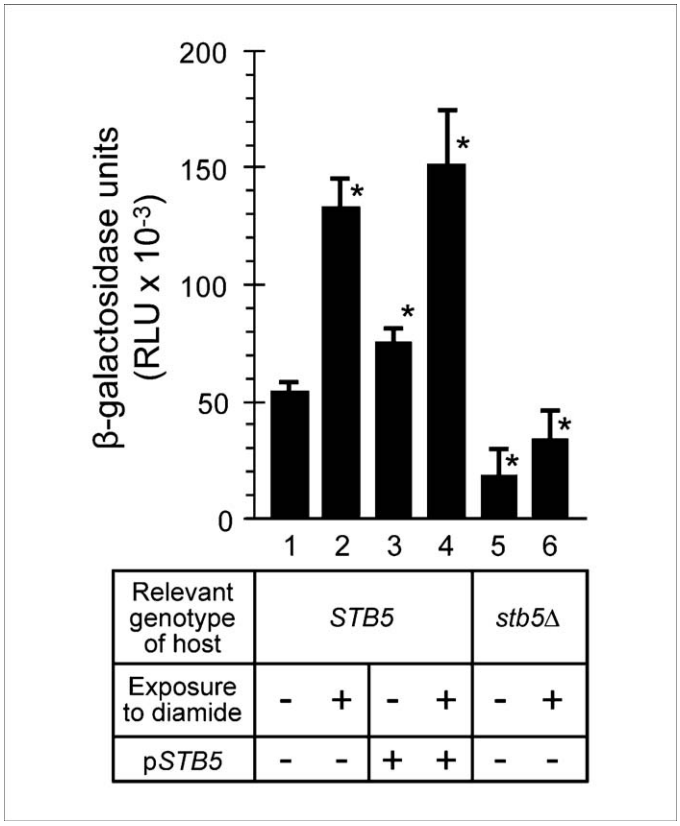

FIGURE 2

Stb5 mediated transcriptional activation of the *YMR315W* promoter in response to diamide induced NADPH depletion. *STB5* and *stb5Δ* cells containing the native 5′-UTR *YMR315W* reporter plasmid, pRH280, were exposed to 1.8 mM diamide for 30 min before measuring β-galactosidase activity (bars 1 and 2 versus 5 and 6). The effect of increased Stb5p (from p*STB5*) on *YMR315W* transcription in diamide-stressed cells was also assayed (bars 3 and 4 versus 1 and 2). β-Galactosidase activities are reported as relative light units (RLU $\times$ 10^{-3}) and are the average values for $\geq$3 independent cultures. Error bars represent the standard deviation. *Value is significantly different from the parent strain, Bar-1 ($P < 0.05$).

highly on the cells ability to maintain NADPH levels. As such, cells with impaired ability to regenerate NADPH show decreased growth upon exposure to diamide. For example, cells lacking *STB5*, which has been suggested to regulate NADPH production, are sensitive to diamide [15].

We tested whether *YMR315W* transcription responded to NADPH depletion that results from exposure to diamide. β-Galactosidase activity was measured from cells containing the *YMR315W::lacZ* reporter plasmid, pRH280, that were exposed to 1.8 mM diamide for 30 min. Exposure of the parent strain (YRH373) to diamide resulted in a 2.6-fold increase in activity (Fig. 2, bar 1 versus 2). The lack of increased transcriptional activity from *stb5*Δ cells showed that Stb5p is required for the increase in activity due to diamide (bar 2 versus 6). As seen above, in the absence of diamide, increased expression of Stb5p from plasmid p*STB5* in BY4727 cells resulted in increased transcription (bar 1 versus 3). Compared to the parent strain exposed to diamide, activity was slightly elevated in diamide-stressed cells also overexpressing Stb5p (bar 2 versus 4); however, this difference was not significant. These results indicate that *YMR315W* transcription is increased in response to diamide exposure and that Stb5p is required for this increase.

Ymr315wp overexpression in BY4727 cells increases diamide resistance and NADPH

Because *stb5*Δ cells are sensitive to diamide, *YMR315W* transcription is induced by diamide, and the increased *YMR315W* transcriptional activity requires Stb5p, we wanted to determine if cells lacking *YMR315W* were sensitive to diamide. To assay diamide sensitivity, cell growth during exposure to varying concentrations of diamide was measured for BY4727 and *ymr315w*Δ cells (strain YRH100). Cells lacking the *STB5* gene (*stb5*Δ cells, strain YRH429) were included for comparison. As expected, *stb5*Δ cells were hypersensitive to diamide (Fig. 3A). Cells lacking Ymr315wp were not different than the parent strain. Since the PPP is the main pathway for NADPH production from glucose, and is still active in the *ymr315w*Δ strain, it was possible that any effect of a *YMR315W* deletion was not observable due to the activity of the PPP.

To further test the ability of Ymr315wp to contribute to NADPH production, we looked at diamide sensitivity in cells expressing elevated levels of Ymr315wp. Increasing NADPH availability has been shown to result in increased resistance to diamide [26]. We hypothesized that diamide resistance would increase if more NADPH was available in cells with increased amounts of Ymr315wp. We also looked at diamide resistance in cells expressing elevated levels of Stb5p. Cells overexpressing Stb5p displayed increased resistance to diamide (Fig. 3A), consistent with its proposed role in NADPH production. Cells overexpressing Ymr315wp were also more resistant to diamide, suggesting a possible role for Ymr315wp in NADPH production.

Since elevated levels of Ymr315wp resulted in increased resistance to diamide, we asked if Ymr315wp could provide NADPH in cells lacking the main pathway for NADPH. *ZWF1* (glucose-6-phosphate dehydrogenase) is the first enzyme in the oxidative branch of the PPP [27]. During growth on glucose, the oxidative branch of the PPP provides the majority of NADPH that is used for reducing power by the glutathione and thioredoxin reductases to protect cells against oxidative stress [2,28]. As expected, cells deleted for the *ZWF1* gene showed increased sensitivity to NADPH stress imposed by diamide exposure (Fig. 3B). Surprisingly, cells lacking both *ZWF1* and *YMR315W* genes were no longer hypersensitive to diamide. Additionally, upon expressing Ymr315wp in the *zwf1*Δ *ymr315w*Δ strain, hypersensitivity to diamide was re-established (Fig. 3B). These results suggested that whileYmr315wp increased diamide resistance in the parent strain, in cells lacking the oxidative branch of the PPP, Ymr315wp expression contributed to diamide sensitivity, possibly due to NADPH depletion.

To test the hypothesis that NADPH availability was responsible for the changes in diamide sensitivity seen in these cells, we compared NADPH levels in diamide resistant and sensitive cells. We first measured NADPH in cells with elevated levels of Ymr315wp and Stb5p, which were shown to have increased resistance to diamide. The level of NADPH in the parent strain was measured at 10.7 ± 1.6 nmol/mg protein (Fig. 4, bar 1). Cells expressing either the *YMR315W* or *STB5* gene from a low-copy vector showed significant increases in NADPH levels compared to wild-type cells (Fig. 4, bar 1 versus 2 and 3). This increase in

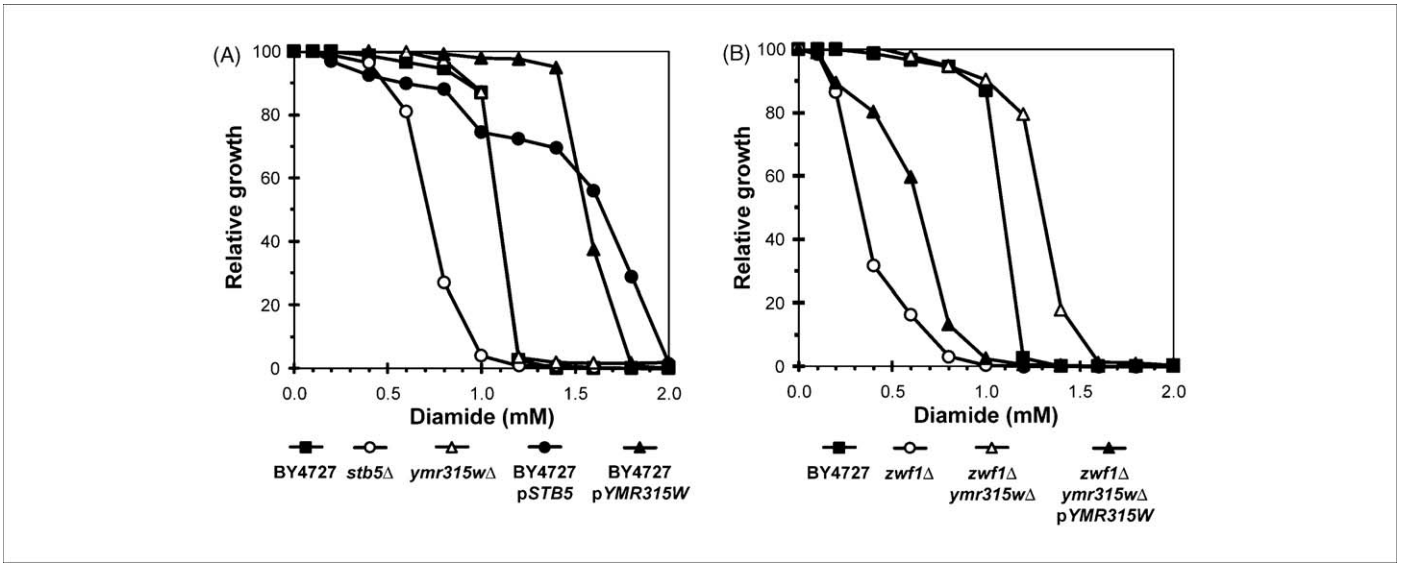

FIGURE 3

Diamide sensitivity assays. Cells from mid log phase growth were diluted into various concentrations of diamide. Relative growth was determined after 44 hours of culture in the presence of diamide. **(A)** The effect of *STB5* and *YMR315W* expression was assayed by measuring the diamide sensitivity of strains either lacking or overexpressing the genes. Elevated expression of Stb5p or Ymr315wp results in increased resistance to diamide. **(B)** The effect of *YMR315W* expression on diamide sensitivity in cells lacking the main source of NADPH (*zwf1Δ* cells) was determined. Expression of *YMR315W* increases diamide sensitivity *zwf1Δ* cells. Results shown are the average values for ≥3 independent cultures. For clarity, error bars are not shown. Standard deviations for each diamide concentration were typically less than 4%, except at concentrations immediately surrounding a rapid decline in percent growth over a narrow diamide concentration range. Standard deviations at these concentrations were increased, but were typically less than 15%. Relative growth was calculated as [100 × (Culture + diamide OD_{660}/culture without diamide OD_{660})].

NADPH is consistent with the observed increased diamide resistance of these strains.

Cells lacking the oxidative branch of the PPP (*zwf1Δ* cells) contained significantly less NADPH (2.4 ± 0.5 nmol/mg, $P = 0.001$) (Fig. 4, bar 1 versus 4). Deletion of native *YMR315W* in the *zwf1Δ* strain restored NADPH to wild-type levels (Fig. 4, bar 4 versus 5). These results are consistent with the difference in diamide sensitivity between the strains and indicated that native Ymr315wp activity is partly responsible for the low levels of NADPH in *zwf1Δ* cells. Expressing Ymr315wp from p*YMR315W* in the *zwf1Δ ymr315wΔ* strain, while resulting in diamide sensitivity, did not significantly change the NADPH level (Fig. 4, bar 5 versus 6). Since we did not see a difference in NADPH levels in these cells, we also compared $NADP^+$ levels between *zwf1Δ ymr315wΔ* cells and *zwf1Δ ymr315wΔ* + p*YMR315W*. $NADP^+$ levels increased 77% ($P = 0.009$) in *zwf1Δ ymr315wΔ* + p*YMR315W* cells compared to the *zwf1Δ ymr315wΔ* strain (data not shown). This latter result indicates that in the *zwf1Δ ymr315wΔ* + p*YMR315W* strain, Ymr315wp increases NADPH oxidation to $NADP^+$, increasing the $NADP^+$/NADPH ratio, resulting in the increased diamide sensitivity observed with this strain.

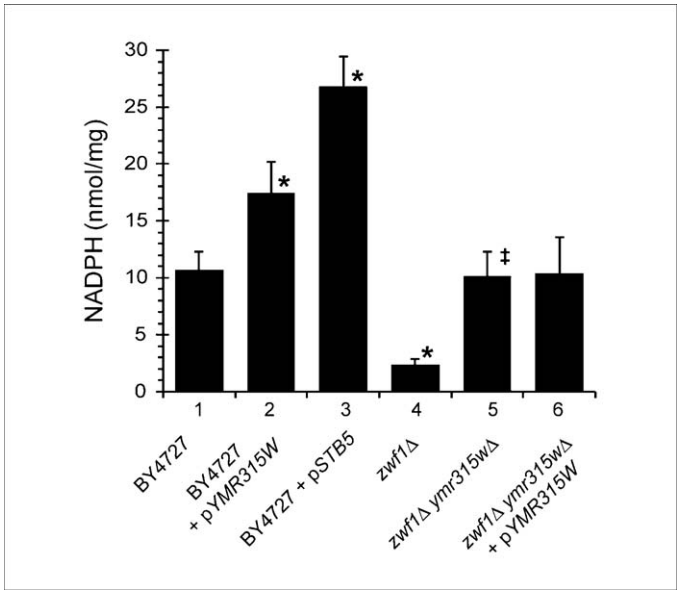

FIGURE 4

Expression of *STB5* and *YMR315W* alters NADPH levels. All cofactor amounts were normalized to the protein content of the lysate used and are reported as (nmol/mg of protein). Results shown are the average values for three cultures. Error bars represent the standard deviation. *Value is significantly different ($P < 0.05$) from BY4727, bar-1. ‡Value is significantly different from *zwf1Δ* strain ($P = 0.004$), bar 4 versus 5.

Ymr315wp is an NADP(H)-specific oxidoreductase

Because expression of Ymr315wp in BY4727 cells increased NADPH levels, but expression in *zwf1Δ* cells increased $NADP^+$ levels, it was possible that Ymr315wp could act as both a reductase and dehydrogenase, depending on the substrate(s) available. To identify substrates for Ymr315wp, recombinant His-tagged protein was purified from bacteria (Fig. 5A) and used to test both reductase and dehydrogenase activities, using either NADP(H) or NAD(H). Because Ymr315wp shows similarity to glucose–fructose oxidoreductase (GFOR) from *Z. mobilis* [12] and xylose dehydrogenase from *H. jecorina* [13], we tested multiple sugar substrates. We concluded that Ymr315wp could function as a reductase and dehydrogenase, with preference for NADP(H) over NAD(H), as activity was only seen when $NADP^+$ or NADPH were used as cofactors (Fig. 5B,C).

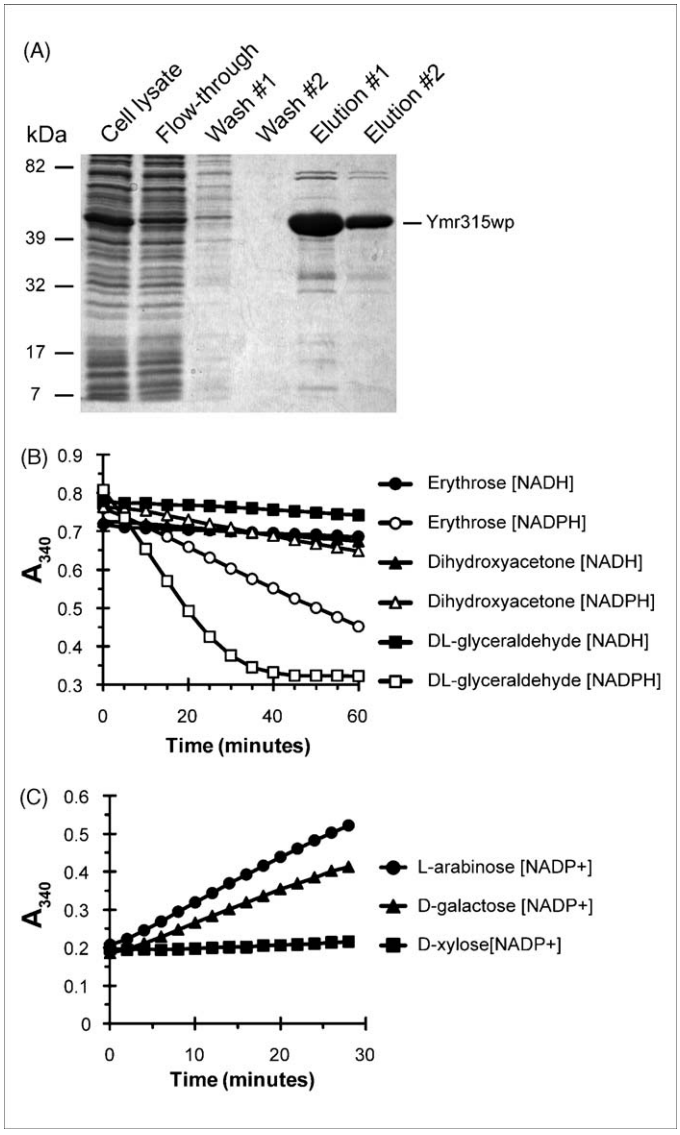

FIGURE 5

Reductase and dehydrogenase activity with recombinant His-tagged Ymr315wp. **(A)** Coomassie-stained gel showing purified recombinant His-tagged Ymr315wp. **(B)** Reduction reactions showing specificity for NADPH over NADH. The highest reductase activity was seen using DL-glyceraldehyde as a substrate and NADPH. Reductase activity was not detected using NADH as a cofactor. **(C)** Oxidation reactions showing activity with L-arabinose and D-galactose. Dehydrogenase activity with these substrates was not seen when NAD^+ was used in place of $NADP^+$ (data not shown).

TABLE 3

Substrate activity with Ymr315w-HISp

	Relative activity
Reduction	
DL-Glyceraldehyde	100
D-Erythrose	26
Dihydroxyacetone	15
Methylglyoxal	11
Diacetyl	n.d.
Acetoin	n.d.
D-Ribose	n.d.
D-Fructose	n.d.
D-Glucose	n.d.
D-Xylose	n.d.
Oxidation	
L-Arabinose	100
D-Galactose	74
D-Xylose	n.d.
D-Glucose	n.d.
D-Arabinose	n.d.
D-Ribose	n.d.
Acetaldehyde	n.d.
Acetoin	n.d.
Butanediol	n.d.
Erythritol	n.d.
Xylitol	n.d.
Glycerol	n.d.
Sorbitol	n.d.

All substrates were tested at 10 mM. Reduction reactions were done at pH 7.0 using 1.0 mM NADPH. Oxidation reactions were at pH 8.0 with 0.15 mM $NADP^+$. No activity was seen using NAD(H). n.d. = not detected.

Ymr315wp was capable of NADPH-dependent reduction of DL-glyceraldehyde and erythrose to glycerol and erythritol, respectively (Table 3). Interestingly, *zwf1*Δ cells reverse flux through the nonoxidative part of the PPP to provide the biomass precursors erythrose-4-P and pentose-5-P [29]. If erythrose-4-P accumulates in *zwf1*Δ cells, the erythrose reductase activity of Ymr315wp could further decrease NADPH levels. To determine if Ymr315wp expression resulted in increased erythritol production, we measured the concentration of extracellular erythritol. Erythritol was not detected in the parent strain or strains not overexpressing Ymr315wp. Extracellular erythritol accumulation was observed using *zwf1*Δ *ymr315w*Δ cells overexpressing Ymr315wp (Table 4). However, we do not believe the amount of erythritol produced in the *zwf1*Δ *ymr315w*Δ + p*YMR315W* strain (0.024 g/g cells) is enough to result in the reversal of diamide sensitivity observed with this strain. A much larger increase was seen with extracellular glycerol. Compared to *zwf1*Δ *ymr315w*Δ cells,

TABLE 4

Product accumulation after 16 hours aerobic growth in YNB + 20 g/l glucose

Strain	Erythritol	Glycerol	Ethanol	Acetate
BY4727	n.d.	0.28 (0.014)	3.5 (0.18)	0.10 (0.002)
***ymr315w*Δ**	n.d.	0.27 (0.005)	3.6 (0.05)	0.10 (0.007)
***zwf1*Δ**	n.d.	0.33 (0.007)	3.5 (0.09)	0.11 (0.004)
***zwf1*Δ *ymr315w*Δ**	n.d.	0.33 (0.006)	3.8 (0.04)	0.11 (0.004)
zwf1*Δ *ymr315w*Δ + p*YMR315W	0.024* (0.004)	0.43* (0.001)	4.9* (0.01)	0.16* (0.006)

Product amounts are given in gram product per gram cells (dry weight) (±SD). n.d. = not detected.

* Values that are significantly different compared to BY4727 at a $P < 0.05$.

extracellular glycerol increased 30% ($P = 0.001$, from 0.33 to 0.43 g/g cells) using *zwf1*Δ *ymr315w*Δ cells overexpressing Ymr315wp (Table 4). It is possible that in *zwf1*Δ cells, NADPH-dependent reduction of glyceraldehyde to glycerol by Ymr315wp contributes to the decrease in NADPH and increased diamide sensitivity. Ethanol and acetate also increased in cells overexpressing Ymr315wp. Acetate increased 45% ($P = 0.007$, from 0.11 to 0.16 g/g cells) in cells overexpressing Ymr315wp. Acetate also increases in *zwf1*Δ cells (engineered to ferment xylose) grown on glucose/xylose mixtures [30]. This increase in acetate, if mediated by the $NADP^+$-specific acetaldehyde dehydrogenase Ald6p, would provide NADPH in cells lacking the oxidative branch of the PPP [30]. In this context, the increase in acetate observed in our study might be an indication of increased need for NADPH in *zwf1*Δ cells overexpressing Ymr315wp, further suggesting an altered redox balance due to Ymr315p expression. These data indicate that in a *zwf1*Δ strain, Ymr315wp, through its NADPH-specific reductase activity, adds to the NADPH deficiency and might explain why deletion of *YMR315W* in a *zwf1*Δ strain restores NADPH and diamide resistance to normal levels.

Conclusions

The above results indicate that in cells with a functional PPP, Ymr315wp is induced by Stb5p in response to diamide, and Ymr315wp can add to the supply of NADPH and increase resistance to diamide. However, in cells lacking the oxidative branch of the PPP due to deletion of the *ZWF1* gene, the presence of Ymr315wp depletes NADPH, resulting in increased sensitivity to diamide. One possible explanation for this difference in activity is that in *zwf1*Δ cells a metabolite increases that, due to its low concentration in wild-type cells, is normally not a substrate for Ymr315wp. In support of this hypothesis, cells lacking *ZWF1* drastically alter metabolic flux to provide NADPH. For example, *zwf1*Δ cells were shown to reverse flux through the nonoxidative part of the PPP to provide erythrose-4-P and pentose-5-P [29]. In this same study, malic enzyme flux was also significantly increased to provide NADPH. *S. cerevisiae* cells also progress through a metabolic cycle (oxidative/reductive cycle) where metabolites, and the genes required for the metabolites, peak at specific times through the metabolic cycle [31]. Metabolic cycling is abolished in *zwf1*Δ cells [32], allowing futile cycling reactions to occur in cells lacking *ZWF1*. Thus, it seems highly probable that Ymr315wp might reduce metabolites (e.g. glyceraldehyde and erythrose) in *zwf1*Δ cells that are not normally substrates for Ymr315wp in *ZWF1* cells.

In summary, we have shown that Ymr315wp is regulated by the transcription factor Stb5p in response to diamide induced NADPH limitation. We have also shown that overexpression of Ymr315wp in BY4727 cells leads to increased diamide resistance and increased NADPH. Purified recombinant Ymr315p showed both reductase and dehydrogenase activities that are specific for NADPH and $NADP^+$. It will be extremely important to identify other substrates for this enzyme as well as identify the sources of NADPH in the *zwf1*Δ *ymr315w*Δ strain, to better understand how cells respond to maintain an adequate supply of NADPH to provide reducing power for biomass formation and oxidative stress resistance, especially when metabolizing biomass-derived pentose sugars from lignocellulosic hydrolysates.

Acknowledgements

We thank Katherine Card for generating the strains used and for the excellent technical assistance throughout this study. We would also like to thank Greg Kennedy for his help evaluating extracellular metabolite concentrations.

References

1 Verduyn, C. *et al.* (1985) Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*. *Biochem. J.* 226, 669–677

2 Bruinenberg, P.M. (1986) The NADP(H) redox couple in yeast metabolism. *Antonie Van Leeuwenhoek*. 52, 411–429

3 Hahn-Hägerdal, B. *et al.* (2001) Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. *Adv. Biochem. Eng. Biotechnol.* 73, 53–84

4 Jeffries, T.W. (2006) Engineering yeasts for xylose metabolism. *Curr. Opin. Biotechnol.* 17, 320–326

5 van Maris, A.J. *et al.* (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *Antonie Van Leeuwenhoek* 90, 391–418

6 Palmqvist, E. and Hahn-Hägerdal, B. (2000) Fermentation of lignocellulosic hydrolysates. II. Inhibitors and mechanisms of inhibition. *Bioresour. Technol.* 74, 25–33

7 Jeppsson, M. *et al.* (2003) The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant *Saccharomyces cerevisiae* strains. *Yeast* 20, 1263–1272

8 Gorsich, S.W. *et al.* (2006) Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes *ZWF1*, *GND1*, *RPE1*, and *TKL1* in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 71, 339–349

9 Pitkänen, J.P. *et al.* (2003) Metabolic flux analysis of xylose metabolism in recombinant *Saccharomyces cerevisiae* using continuous culture. *Metab. Eng.* 5, 16–31

10 Salusjärvi, L. *et al.* (2008) Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 7, 18

11 Salusjärvi, L. *et al.* (2003) Proteome analysis of recombinant xylose-fermenting *Saccharomyces cerevisiae*. *Yeast* 20, 295–314

12 Kingston, R.L. *et al.* (1996) The structure of glucose–fructose oxidoreductase from *Zymomonas mobilis*: an osmoprotective periplasmic enzyme containing non-dissociable NADP. *Structure* 4, 1413–1428

13 Berghall, S. *et al.* (2007) Identification in the mould *Hypocrea jecorina* of a gene encoding an NADP(+): D-xylose dehydrogenase. *FEMS Microbiol. Lett.* 277, 249–253

14 Dambe, T.R. *et al.* (2006) Crystal structure of NADP(H)-dependent 1,5-anhydro-D-fructose reductase from *Sinorhizobium morelense* at 2.2 Å resolution: construction of a NADH-accepting mutant and its application in rare sugar synthesis. *Biochemistry* 45, 10030–10042

15 Larochelle, M. *et al.* (2006) Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. *Mol. Cell. Biol.* 26, 6690–6701

16 Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual* (3rd edn), Cold Spring Harbor Laboratory Press

17 Gietz, R.D. and Schiestl, R.H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* 2, 31–34

18 Amberg, B.C. *et al.* (2005) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual* (2005 edn), Cold Spring Harbor Laboratory Press

19 Christianson, T.W. *et al.* (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110, 119–122

20 Brachmann, C.B. *et al.* (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14, 115–132

21 Myers, A.M. *et al.* (1986) Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. *Gene* 45, 299–310

22 Nguyen, D.T. *et al.* (2001) Multiple Yap1p-binding sites mediate induction of the yeast major facilitator *FLR1* gene in response to drugs, oxidants, and alkylating agents. *J. Biol. Chem.* 276, 1138–1145

23 Harbison, C.T. *et al.* (2004) Transcriptional regulatory code of a eukaryotic genome. *Nature* 431, 99–104
24 Kosower, N.S. *et al.* (1969) Diamide, a new reagent for the intracellular oxidation of glutathione to the disulfide. *Biochem. Biophys. Res. Commun.* 37, 593–596
25 Grant, C.M. *et al.* (1996) Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation. *Mol. Microbiol.* 21, 171–179
26 Ralser, M. *et al.* (2007) Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress. *J. Biol.* 6, 10
27 Nogae, I. and Johnston, M. (1990) Isolation and characterization of the *ZWF1* gene of *Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase. *Gene* 96, 161–169
28 Minard, K.I. and McAlister-Henn, L. (2005) Sources of NADPH in yeast vary with carbon source. *J. Biol. Chem.* 280, 39890–39896
29 Blank, L.M. *et al.* (2005) Large-scale ^{13}C-flux analysis reveals mechanistic principles of metabolic network robustness to null mutations in yeast. *Genome Biol.* 6, R49
30 Jeppsson, M. *et al.* (2002) Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. *Appl. Environ. Microbiol.* 68, 1604–1609
31 Tu, B.P. *et al.* (2005) Logic of the yeast metabolic cycle: temporal compartmentalization of cellular processes. *Science* 310, 1152–1158
32 Tu, B.P. *et al.* (2007) Cyclic changes in metabolic state during the life of a yeast cell. *Proc. Natl. Acad. Sci. U. S. A.* 104, 16886–16891